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Activin-Induced Factors Maintain *goosecoid* Transcription through a Paired Homeodomain Binding Site

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Previous studies in both *Xenopus* and zebrafish have shown that *goosecoid* is one of the first genes to be transcribed at the onset of gastrulation. *Goosecoid* transcription still initiates when embryos are treated with protein synthesis inhibitors, indicating that it is mediated by preexisting factors and suggesting that *goosecoid* transcription is immediately downstream of the maternal mesoderm-inducing signal. However, *goosecoid* transcription continues long after this maternal signal has ceased to be active, indicating that there are mechanisms to maintain activin-induced transcription. Our study has focused on understanding the factors required to maintain this transcription. We have defined an element within the zebrafish *goosecoid* promoter that is sufficient for activin inducibility in both *Xenopus* and zebrafish embryos. This element, the goosecoid activin element, interacts with two developmentally regulated proteins from *Xenopus* embryos. A maternal protein interacts through cleavage stages until the midblastula transition, and a second protein binds from the onset of gastrulation. The second protein is zygotically expressed, and its binding is required for activin inducibility in our assay system. We suggest that the zygotic protein we have identified is a good candidate to be involved in the maintenance of *goosecoid* transcription. Furthermore, this zygotic protein is likely to contain a paired class homeodomain since a consensus binding site for such proteins is present within the goosecoid activin element and is essential for its function.

Key Words: **transcription; regulation; *Xenopus;* dorsal mesoderm; activin.**

INTRODUCTION

The dorsal mesoderm of the vertebrate gastrula is capable of inducing and organizing dorsal structures that lie along the embryonic body axis, including neural tissue and muscle (Spemann, 1938; Beddington, 1994; Harland and Gerhart, 1997; Heasman, 1997). This was first demonstrated in amphibians when the dorsal mesoderm of one gastrula was transplanted to the ventral side of a second, causing the host embryo to develop a secondary axis. Subsequent studies in other vertebrates established that the central role of the amphibian dorsal mesoderm, or "organizer," is likely to be conserved throughout the vertebrates. We are therefore interested in understanding the events involved in specifying and maintaining the identity of this tissue.

The process of dorsal mesoderm induction is best understood in *Xenopus laevis*, in which blastomere transplantation experiments indicate that a signal from the dorsal vegetal cells can induce dorsal mesoderm in the overlying marginal zone (Dale *et al.*, 1985; Gimlich, 1986; Gimlich and Gerhart, 1984; Nieuwkoop, 1973). This signal is active during blastula stages, before the onset of zygotic transcription, and is therefore at least partially derived from maternally deposited factors (Jones and Woodland, 1987), though zygotically produced factors make an important contribution (Wylie *et al.*, 1996). Dorsal vegetal cells lose their inducing potential after the onset of zygotic transcription, and ectodermal cells lose their ability to respond to these vegetal signals (Gurdon *et al.*, 1985; Jones and Woodland, 1987; Green *et al.*, 1990). However, since the dorsal mesoderm is stably determined there must be mechanisms to maintain its identity after the initial signal is inactive. We are interested in understanding the molecular basis of the mechanism for maintaining dorsal mesodermal identity.

Secreted proteins are likely to initiate dorsal mesoderm induction, and some candidates are known to have the

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expected activities. Activities that act through ALK4 (the activin type 1b receptor) are strong candidates, since blocking ALK4 activity results in the selective loss of mesoderm (Chang *et al.,* 1997; Hemmati-Brivanlou and Melton, 1992). A candidate ligand is activin, which is a member of the transforming growth factor-β (TGF-β) superfamily. When synthetic activin mRNA is injected into ventral blastomeres of *Xenopus* embryos a partial secondary body axis can develop (Thomsen *et al.,* 1990). Activin can also induce ectodermal explants to transcribe markers of dorsal mesoderm, such as *goosecoid* (Ariizumi *et al.,* 1991; Green and Smith, 1990; Green *et al.,* 1992; Thomsen *et al.,* 1990). These experiments therefore establish the requirement for an activin-like signal for mesoderm induction. While activin has activities expected of a maternal mesoderm-inducing signal, it has not been shown to be localized to the dorsal vegetal cells of the *Xenopus* blastula. Instead, an early wnt-like signal is likely to provide the essential dorsal information, and when this activity is superimposed on the activin-like signal, dorsal mesoderm is induced (Harland and Gerhart, 1997; Heasman, 1997). Transcripts of other TGF-β family members with activities similar to those of activin, such as Vg1, Xnr1, and Xnr2, are known to be present in blastula-stage embryos and may provide the required activin-like signal (Jones *et al.,* 1995; Thomsen and Melton, 1993).

Goosecoid is a homeobox gene capable of inducing the formation of a secondary body axis when ectopically expressed on the ventral side of a *Xenopus* embryo (Cho *et al.,* 1991; Steinbeisser *et al.,* 1993). Its transcripts are first detected in the dorsal mesoderm of the late blastula in both *Xenopus* and zebrafish (Stachel *et al.,* 1993) and, as such, it is one of the first genes to be expressed in the dorsal mesoderm. *Goosecoid* transcription is induced by activin and this transcription can be activated in the absence of protein synthesis. Thus *goosecoid* is expected to be a direct target of the maternal dorsal mesoderm-inducing signal. Additionally, *goosecoid* transcription continues throughout gastrulation, indicating that mechanisms exist to maintain expression after maternal signals are lost or ineffective. Analysis of *goosecoid* transcription therefore allows the study of molecules required for the initial induction and those required for maintenance of transcription.

Previous studies in *Xenopus* on the transcriptional regulation of *goosecoid* and another activin-inducible gene, *Mix,* have identified sequence elements and binding proteins implicated in the initial protein synthesis independent induction of transcription (Huang *et al.,* 1995; Watabe *et al.,* 1995). A sequence element required for activin-inducibility, the activin-response element (ARE), has been identified within the *Mix* promoter (Huang *et al.,* 1995). A complex which interacts with this element, and which is required for activin induction, contains a member of the vertebrate Smad family, Smad2, and a novel winged-helix protein, FAST1 (Chen *et al.,* 1996). Studies on the *goosecoid* promoter have identified two upstream elements implicated in the regulation of its transcription, one of which, the distal element (DE), confers activin responsiveness upon heterologous promoters (Watabe *et al.,* 1995). This response is independent of protein synthesis inhibitors, indicating that it may be involved in response to the maternal mesoderm-inducing signal. So far there have been no reports of elements involved in the maintenance of dorsal mesodermal identity.

Previously, Joore *et al.,* (1996) defined elements from a zebrafish *goosecoid* promoter that confer an immediate-early response to activin. These elements map far upstream of the initiation site. Here we define a proximal element from the zebrafish *goosecoid* promoter which is required for the activin response of a truncated zebrafish promoter and which is similar to the activin-responsive DE identified in *Xenopus.* We have detected two developmentally regulated complexes which interact specifically with this element. A maternal complex present in cleavage-stage embryos is no longer detected by the midblastula transition. A second complex is zygotic and is detected throughout gastrula stages. This second complex can be induced by activin and binding of the complex correlates with the activin-inducibility of mutant *goosecoid* promoters. While a previous study on a similar element (the DE) in the *Xenopus* promoter suggested that this element confers an immediate-early response (Watabe *et al.,* 1995), we find that the zebrafish element has properties more consistent with a critical role in the maintenance of activin-induced transcription. This activin-responsive element matches the consensus binding site for paired-class homeodomains, suggesting that a member of this class of transcription factors may directly regulate *goosecoid* transcription.

MATERIALS AND METHODS

Plasmids

To test *goosecoid* promoter elements, a 4.4-kb *Eco*RI fragment of a zebrafish *goosecoid* genomic clone that extends upstream of an *Eco*RI site in the *goosecoid* 5′ untranslated region (UTR) was ligated into the *Eco*RI site of pBluescript (Stratagene). The restriction sites in the polylinker of pBluescript facilitated subsequent subcloning of parts of this *Eco*RI fragment into the polylinker of pGL2basic (Promega) to generate the constructs shown in Fig. 2a. A 4.0-kb *Xho*I fragment was ligated into the *Xho*I site of pGL2basic to make p4.0gsclux. The 1.9-kb *Eco*RV/*Bam*HI fragment was ligated into *Sma*I/*Bgl*II-digested pGL2basic to construct p1.9gsclux. The *Cla*I/*Bam*HI fragment was ligated into *Sma*I/*Bgl*II-digested pGL2basic to construct p0.6gsclux. The 0.3 kb *Hinc*II/*Bam*HI fragment was cloned into *Sma*I/*Bgl*II-digested pGL2basic to construct p0.3gsclux.

A different version of this plasmid, p0.3FDgsclux, was used to generate exonuclease III deletions. p0.3FDgsclux was constructed by ligating the 0.3-kb *Hinc*II/*Bam*HI fragment into *Nhe*I (blunted)/*Bgl*II-digested pGL2basic. This construct was digested to completion with *Kpn*I and *Mlu*I, and short 5′ deletions into the *goosecoid* sequence were made using exonuclease III. The endpoints of the exonuclease III deletions were sequenced and are indicated in Fig. 1.

In pMSVCAT the murine sarcoma virus LTR promoter is up-

stream of the gene for chloramphenicol acetyl transferase (CAT) (Harland and Misher, 1988). This promoter is ubiquitously expressed in *Xenopus* during gastrulation.

pXeXβgal was constructed by ligating a 5-kb fragment encoding nuclear β-galactosidase (Picard *et al.,* 1988) downstream of the EF1α promoter in pXeX (Johnson and Krieg, 1994) (pXeXβgal was a kind gift from F. Mariani). The plasmid pGlobin-lux was constructed by amplifying the *Xenopus β-globin* promoter (−471 to +74) from pG2XG using oligonucleotides with engineered *Hin*dIII ends. The *globin* promoter was then ligated into the *Hin*dIII site in the pGL2basic polylinker. pG2XG contains the region −471 to +74 of the *Xenopus β-globin* promoter from pXβ (Krieg and Melton, 1985) ligated into pGEM2 and was a kind gift from P. Krieg.

To construct p2xGAEglobin-lux, the region of the *goosecoid* promoter from −189 to −119 was amplified using primers GAE5′#1 and GAE3′#1 (see *Gel Retardation Assay* for sequence). The products were digested with *Spe*I and *Hin*dIII, ligated together, and then subcloned into *Nhe*I/*Bgl*II-digested pGlobin-lux. Sequencing showed that both clones tested contained two head-to-tail copies of the region from −189 to −119. Point mutations were introduced into p0.3FDgsclux by single-stranded oligonucleotide-directed mutagenesis (Kunkel, 1985; Sambrook *et al.,* 1989). The oligonucleotides used to direct synthesis of the mutant strand are indicated (Fig. 6a). These constructs were sequenced to confirm that the mutations had been correctly introduced.

The goosecoid activin element (GAE) in p4.0gsclux was replaced with a *Bam*HI site using single-stranded oligonucleotide-directed mutagenesis (Kunkel, 1985; Sambrook *et al.,* 1989). The oligonucleotide used to direct synthesis was CTGTGTATTGAGAATCAGGATCCATGAGTTATTTGCTGACT.

RNA Synthesis

Synthetic capped mRNA was transcribed *in vitro* from cloned SP6 promoters using the Message Machine Kit (Ambion). Activin βB was transcribed from the SP6 RNA polymerase promoter of pSP64T (Thomsen *et al.,* 1990). *Xenopus otx* genes were isolated as described (Lamb *et al.,* 1993). *Xotx30* is an *otx2* homologue (Lamb and Harland, 1995), and *Xotx33* is related to *otx2,* but is expressed in a distinct pattern in the tadpole, with prominent expression in the cement gland of tailbud tadpoles and in the retina of more mature tadpoles. In our hands, neither of these *otx2* relatives is an efficient inducer of cement gland, unlike other *otx2* transcripts. *Xfkh1* was provided by Milan Jamrich (Dirksen and Jamrich, 1992), *Xlim1* was provided by Igor Dawid (Taira *et al.,* 1992), *Mix1* was provided by Fred Rosa (Rosa, 1989), and *Xanf1* was provided by Andrey Zaraisky (Zaraisky *et al.,* 1995). The coding regions were subcloned into CS2+ (Turner and Weintraub, 1994) and templates were transcribed with SP6 RNA polymerase after digestion with *Not*I. *Siamois* was from John Gurdon and was transcribed from the T3 promoter of pRN3-siamois (Lemaire *et al.,* 1995).

Promoter Assays

To test promoter–reporter constructs *Xenopus* embryos were injected at the single-cell stage with 10 pg test plasmid and 50 pg pMSVCAT in a 10-nl volume. Half of the injected embryos were subsequently injected with 100 pg synthetic activin mRNA. Zebrafish were injected between the 1- and the 16-cell stage with 50 pg test plasmid and 50 pg pXeXβgal in a volume of 1 nl. Samples injected into zebrafish were mixed with phenol red so injected embryos were distinct. Some of the samples contained synthetic activin mRNA at 50 pg/nl as a source of activin ligand.

The amount of luciferase present in an embryo extract from either *Xenopus* or zebrafish was determined using a luciferase assay system (Promega), and results were quantified using a scintillation counter. In each experiment approximately 30 embryos were used to make the extracts, and 2 embryo equivalents were used in the assay. All measurements of luciferase activity were normalized to expression of a different reporter from a ubiquitously expressed promoter. In the *Xenopus* assays, the luciferase measurements were normalized to the amount of CAT activity expressed from the pMSVCAT construct, which was co-injected with the test constructs in all experiments and quantified as described (Neumann *et al.,* 1987). In the zebrafish assays, luciferase measurements were normalized to the amount of β-galactosidase activity expressed from the pXEXβgal construct, which was co-injected in all experiments and quantified as described (Herbomel *et al.,* 1984; Sambrook *et al.,* 1989).

The results presented in Fig. 6f show that there is more transcription from the *goosecoid* promoter fragment on the dorsal than the ventral side of the embryo. In the experiments presented in Fig. 2 the reporter constructs are injected into the animal pole of single-cell embryos. However, variable spread of DNA into the dorsal or ventral marginal zones may lead to some activation of transcription from the promoters by endogenous signals and lead to some variability in the results. To address this problem experiments have typically been repeated five times, and either the results are presented in a table (as in Table 1) or the results of a representative assay are shown.

Gel Retardation Assays

The probes 71GAE, 46L, and 46R were generated by polymerase chain reaction (PCR) using the following oligonucleotides as primers: GAE5′#1, CATACTAGTGGAACTAAATTATTTGTTTC; GAE5′#2, CATACTAGTCAGCAAATAACTTAATC; GAE3′#1, CTGAAGCTTACCGATCTGTGTATTGAG; and GAE3′#2, CTGAAGCTTGATTAAGTTATTTGCTGAC. The plasmid p0.3gsclux was used as template and radiolabeled nucleotide was included in the reaction. 71GAE was amplified between GAE5′#1 and GAE3′#1. 46L was amplified between GAE5′#1 and GAE3′#2. 46R was amplified between GAE5′#2 and GAE3′#1. The oligonucleotides were designed with 5′ restriction sites for cloning purposes, and results of gel retardation assays were the same whether these ends were removed or not. The sequences of probes 30L and 30R are shown in Fig. 3a. These probes were synthesized by end-labeling a 30-mer representing the top strand and annealing it to a slight excess of the bottom strand. All probes were electrophoresed through 10% polyacrylamide gels in 1× TAE. Gel slices containing the probe were cut from the gel and probe was eluted from the slice and precipitated. PCR probes were resuspended at 100 pg/μl, and end-labeled probes were resuspended at 1 ng/μl.

Protein extracts were made by homogenizing embryos in chilled Dignam C (Dignam *et al.,* 1983) containing 50 mM KCl and 1 mM phenylmethylsulfonyl fluoride (5 μl of buffer per embryo) and microfuging for 10 min at 4°C (Snape *et al.,* 1990). The supernatant was removed, avoiding the floating lipid layer and the pellet.

In the binding reactions 11.5 μl extract (the supernatant) was preincubated with 1 μl poly(dI–dC):poly(dI–dC) (Sigma) (0.5 mg/ml) and 1.5 μl 0.5 M NaCl. After 10 min at room temperature 1 μl of probe was added and incubated for a further 15 min at room temperature. The mixture was electrophoresed through 4% polyacrylamide (60:1) in 50 mM Tris–Cl, 380 mM glycine, 2 mM EDTA at 180 V for 2–3 h at 4°C. Glycerol present in the extraction buffer

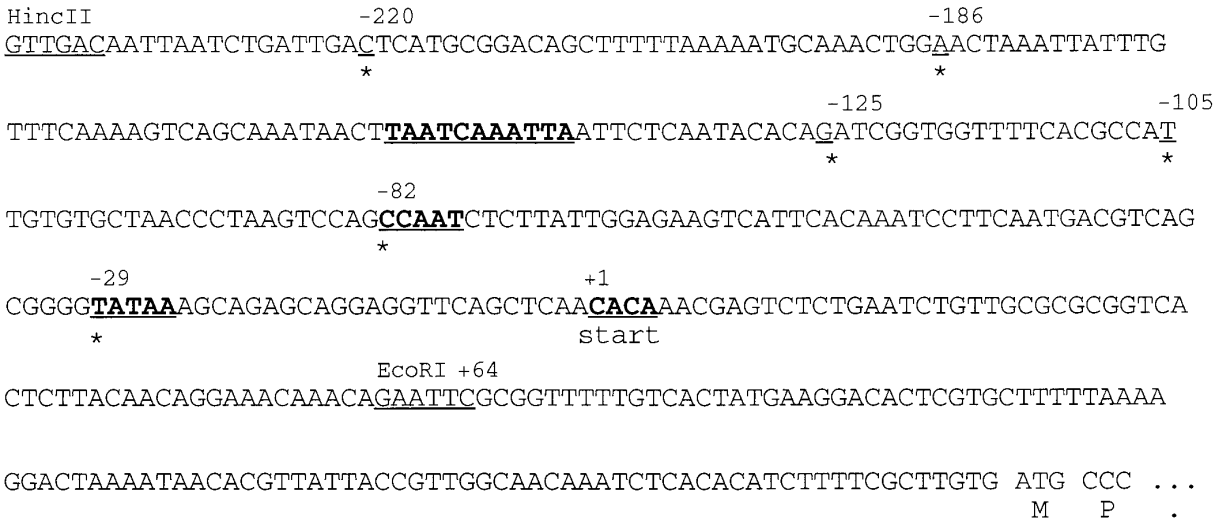

FIG. 1. DNA sequence of the *goosecoid* promoter region. Transcription starts at the CACA at position +1, as determined by RNase protection and primer extension. The TATA and CCAAT boxes, and the paired-class homeodomain binding site (TAATCAAATTA), are highlighted. The endpoints of the exonuclease III deletions are indicated by underlining and an asterisk.

was sufficient to sink the reaction mixtures into the gel wells. A vacant lane on the gel was loaded with dyes to monitor the progression of electrophoresis.

For experiments in which zygotic transcription was inhibited, 10 nl α-amanitin (100 μg/ml) was injected into *Xenopus* embryos at the single-cell stage. This concentration inhibits RNA polymerase II but does not inhibit other RNA polymerases (Gurdon and Brown, 1978; Melton and Cortese, 1979).

In competition experiments competitor DNA was added at the preincubation step. 71GAE and 46L were generated by PCR using the oligonucleotides described above. The unrelated fragment used in competition experiments was generated using oligonucleotides TGTATCTTATGGTACTGTAACTG and CTCTAGAGGATAGAATGG, which flank the pGL2basic polylinker to amplify a 153-bp fragment of the polylinker. Gel retardation assays to detect binding of the activin-inducible factor which binds upstream of the *Mix2* gene were carried out using previously described methods (Huang *et al.*, 1995).

RESULTS

Identification of the goosecoid Activin Element

A zebrafish genomic library was screened with a *goosecoid* cDNA (Stachel *et al.*, 1993), and a clone was isolated. The sequence immediately upstream of the *goosecoid* cDNA is shown (Fig. 1). The transcription start site was mapped by RNase protection and primer extension (data not shown), and both assays indicated that transcription started at the CACA. TATA and CCAAT boxes are present at −29 and −82, respectively, relative to the transcription start site.

Sequence elements which control the transcriptional regulation of a gene often lie upstream of the transcribed sequences. For this reason we isolated a 4-kb fragment that extends upstream of an *Eco*RI site in the *goosecoid* 5′ UTR and ligated it upstream of a luciferase reporter gene. To determine whether this fragment contained sequences sufficient for activin inducibility we injected this construct into zebrafish and *Xenopus* cleavage-stage embryos, either alone or along with synthetic activin mRNA. The embryos efficiently translate such injected mRNA, and so this is a good way to provide the embryo with a large dose of activin protein. The embryos were then harvested at late gastrulation (a stage at which the endogenous *goosecoid* gene is being actively transcribed), and the amount of luciferase activity present in the extracts was quantified. Extracts from activin mRNA-injected embryos contained in excess of 100-fold more luciferase activity than extracts from the embryos which had not received activin mRNA (first row of Table 1 and the first bar of the graph in Fig. 2b).

To identify specific sequence elements required for activin inducibility, fragments of the *goosecoid* upstream region were sequentially deleted from the 5′ end, and the deletion constructs were tested for their ability to support activin-induced transcription in the same assay. Although we often observed a small reduction in transcription with successive deletions, the most dramatic and reproducible effect was observed when the sequences between −185 and −125 were deleted (Table 1 and Fig. 2b). These results indicate that the 5′ boundary of an activin-responsive element lies between 186 and 125 bp upstream of the transcription start site. In all assays, results were similar in both *Xenopus* and zebrafish embryos (Figs. 2c and 2d), indicating that the same element can function in both organisms.

TABLE 1

Deletion endpoint	Experiment 1	2	3	4	5	6	7	8
4.0	65	69.1	42	—	—	3634.4	97.1	607.6
1.9	—	28.5	30.6	—	—	333.6	106.9	150.2
0.6	—	5.9	10.6	—	—	132.9	107.9	91.3
0.3	8	7.1	80.6	79	117.6	213.8	40.7	36.9
0.220	—	—	—	565	—		20.2	83.5
0.186	41	—	—	207	656.9		27.1	90.9
0.125	0.25	—	—	0.4	0.5		0.7	0.2
0.101	—	—	—	2.7	—		5.2	0.5
0.092	—	—	—	0.9	0.3		0.9	0.3

Note. The 5′ end of an activin-responsive element is located between −185 and −125 of the *goosecoid* transcription start site. Results of luciferase reporter assays are presented as the fold increase in activity in activin mRNA-injected gastrulae compared to gastrulae that have not received activin mRNA. The 5′ endpoints of the promoter fragments are indicated in the first column and the results of eight separate experiments are presented in the following eight columns.

To test whether sequences immediately downstream of −186 were sufficient to confer activin inducibility upon a heterologous promoter, two copies of the region between −189 and −119 were ligated upstream of a basal *globin* promoter fused to the luciferase gene. Transcription from the *globin* promoter alone is not induced by activin, but two tandem copies of the region from −189 to −119 were sufficient to confer activin inducibility upon this promoter (Fig. 2e). We call the element defined by this experiment the goosecoid activin element.

An Activin-Inducible Protein Interacts with the GAE

Transcription is often regulated by controlling the binding of a transcription factor to a specific promoter element. To identify potential transcription factors involved in the activin response of the *goosecoid* gene, we performed gel retardation assays using radiolabeled copies of the 71-bp sequence from −189 to −119 (71GAE) as probe. We detected robust binding activity in *Xenopus* embryo extracts and did not pursue biochemistry with zebrafish embryo extracts. Extracts were prepared from uninjected or activin mRNA-injected gastrulae and then incubated with probe; the mixture was then subjected to gel electrophoresis. In these gels, complexes of probe and bound protein migrate more slowly than unbound probe. Several retarded complexes were detected, one of which was activin inducible (arrows in left-hand panel of Fig. 3b). We named this complex the GAE binding protein 1, or GAEBP1. To determine whether GAEBP1 binds the GAE sequence specifically we compared the ability of various DNA sequences to disturb this complex. Binding of GAEBP1 can be disturbed efficiently with 100-fold molar excess of unlabeled GAE, but not with 100-fold molar excess of an unrelated sequence, indicating that GAEBP1 binds specifically to the GAE sequence (Fig. 3c). We divided the GAE into smaller pieces (Fig. 3a) and used these as probes in gel retardation assays to determine the shortest fragment capable of binding this activin-inducible complex. Fragment 30L, containing the sequence from −134 to −163, is the smallest of these probes that can efficiently bind GAEBP1 (Fig. 3b).

Developmental Time Course of GAEBP1 Formation

If GAEBP1 is involved in the activation or maintenance of *goosecoid* transcription, then it should only bind the GAE while *goosecoid* is being actively transcribed. We made protein extracts from embryos harvested at different developmental stages to determine at what stages GAEBP1 binding was detectable. Extracts were made from both uninjected and activin mRNA-injected embryos, and a gel retardation assay was performed. GAEBP1 activity was first detected in the early gastrula (stage 10) and persisted through gastrulation (Fig. 4a). The complex is detected at the same stages but is induced to about 15-fold higher levels in activin mRNA-injected embryos. A maternal complex involved in initiating the transcriptional response of the *Mix2* gene to activin, known as the activin-response factor (ARF), binds an activin-responsive element within the *Mix2* promoter (Huang *et al.*, 1995). ARF can be detected in early blastulae (stage 6), peaks in abundance at late blastula, and declines by early gastrulation (stage 10+). In contrast to ARF, the kinetics of GAEBP1 binding are more consistent with a role in the maintenance of *goosecoid* transcription in the embryo than with a role in initiating transcription.

A second developmentally regulated complex was detected, GAE binding protein 2 (Fig. 4a). GAEBP2 is present in cleavage-stage embryos and is gone by the midblastula transition. Its presence is unaffected by injection of activin mRNA. Inclusion of unlabeled GAE in the binding reaction disturbed formation of the complex, whereas unrelated sequences did not (Fig. 4b). This result indicates that GAEBP2, like GAEBP1, binds the GAE specifically. How-

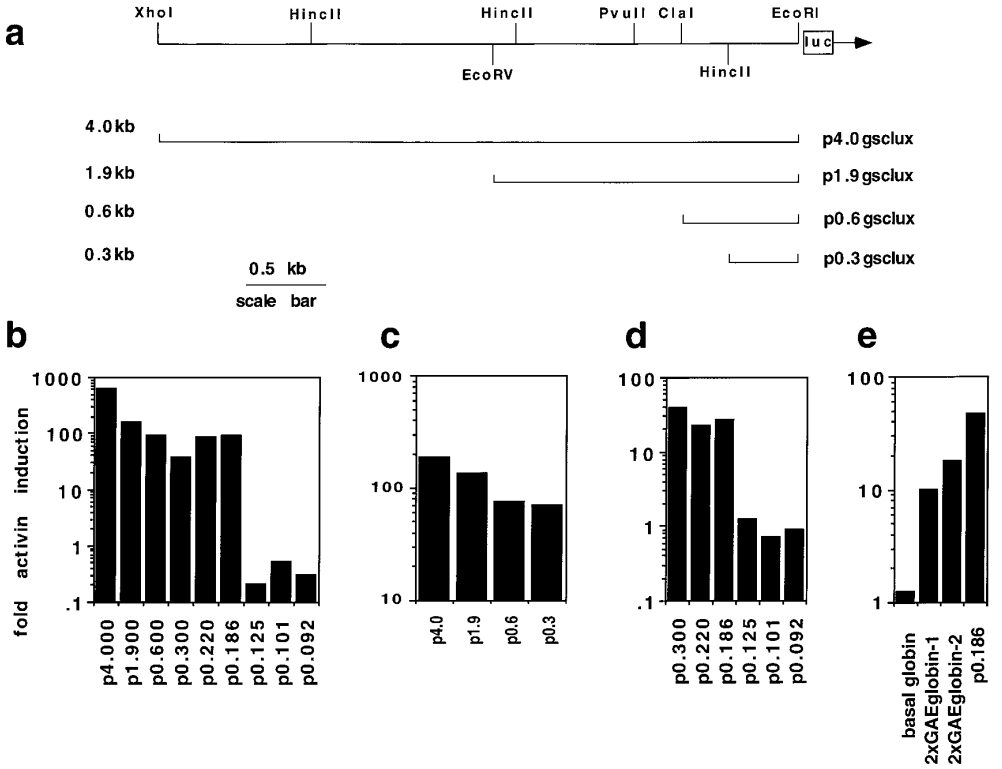

FIG. 2. Mapping an activin-response element within the *goosecoid* promoter. (a) Restriction map of the 4 kb upstream of the *goosecoid* gene and the deletion constructs used to map the activin-responsive element. (b) The activin inducibility of these constructs was tested in *Xenopus* embryos (see also Table 1) and a representative experiment is shown. The responsiveness of each construct is measured as the amount of luciferase activity present in gastrula embryos co-injected with the construct and activin mRNA, divided by the amount of luciferase activity present in gastrula embryos injected with the construct alone (see Materials and Methods for further details). The length of the promoter fragment present in each construct is indicated, i.e., p4.000 (p4.0gsclux) contains 4 kb and p0.220 contains 220 bp upstream of the transcription start site. Results are presented on a logarithmic scale. A dramatic loss of inducibility was detected when the sequences between −186 and −125 were deleted, defining the goosecoid activin element. (c) An example of an assay in which the longer deletion constructs were assayed in zebrafish is shown. In all repeats of this experiment all of these constructs were highly inducible in zebrafish. (d) An example of an assay in zebrafish in which the shorter deletion constructs generated by exonuclease III digestion were tested in zebrafish is shown. The results from the zebrafish assays indicated that the same region of the promoter required for activin-inducibility in *Xenopus* is required in zebrafish. (e) The pGlobin-lux construct has a minimal *globin* promoter upstream of the luciferase reporter gene. This promoter is not activin inducible, but ligation of two tandem copies of the region between −189 and −119 upstream of the *globin* promoter is sufficient to confer activin inducibility upon this promoter. Two different clones, each of which contain two head-to-tail copies of this region, were tested. This assay was carried out three times in *Xenopus* embryos and the results of one representative experiment are presented.

ever, the presence of GAEBP2 in cleavage stages, and its subsequent disappearance during gastrula stages, are more consistent with a repressive role in *goosecoid* expression than the activating role likely for GAEBP1.

GAEBP1 Is Zygotically Transcribed

Goosecoid is transcribed in response to activin in the absence of protein synthesis, indicating that the proteins required for initiating its transcription are already present in the embryo (Cho *et al.*, 1991; Steinbeisser *et al.*, 1993). We tested whether GAEBP1 was present in extracts from embryos injected with α-amanitin, an inhibitor of transcription. If GAEBP1 (or its mRNA) is present before the onset of transcription in the embryo, and its binding (or translation) activated by activin, we would expect to see GAEBP1 binding induced even in the presence of an inhibitor of transcription. However, we found that formation of GAEBP1 is inhibited by α-amanitin, indicating that it is expressed from a zygotic transcript (Fig. 5a). To test whether the concentration of α-amanitin used was nonspecifically toxic to the formation of DNA binding complexes, we tested whether the α-amanitin-injected embryos were still capable of activating the ARF, which binds upstream of the *Mix2* gene (Huang *et al.*, 1995). ARF is maternally supplied and is

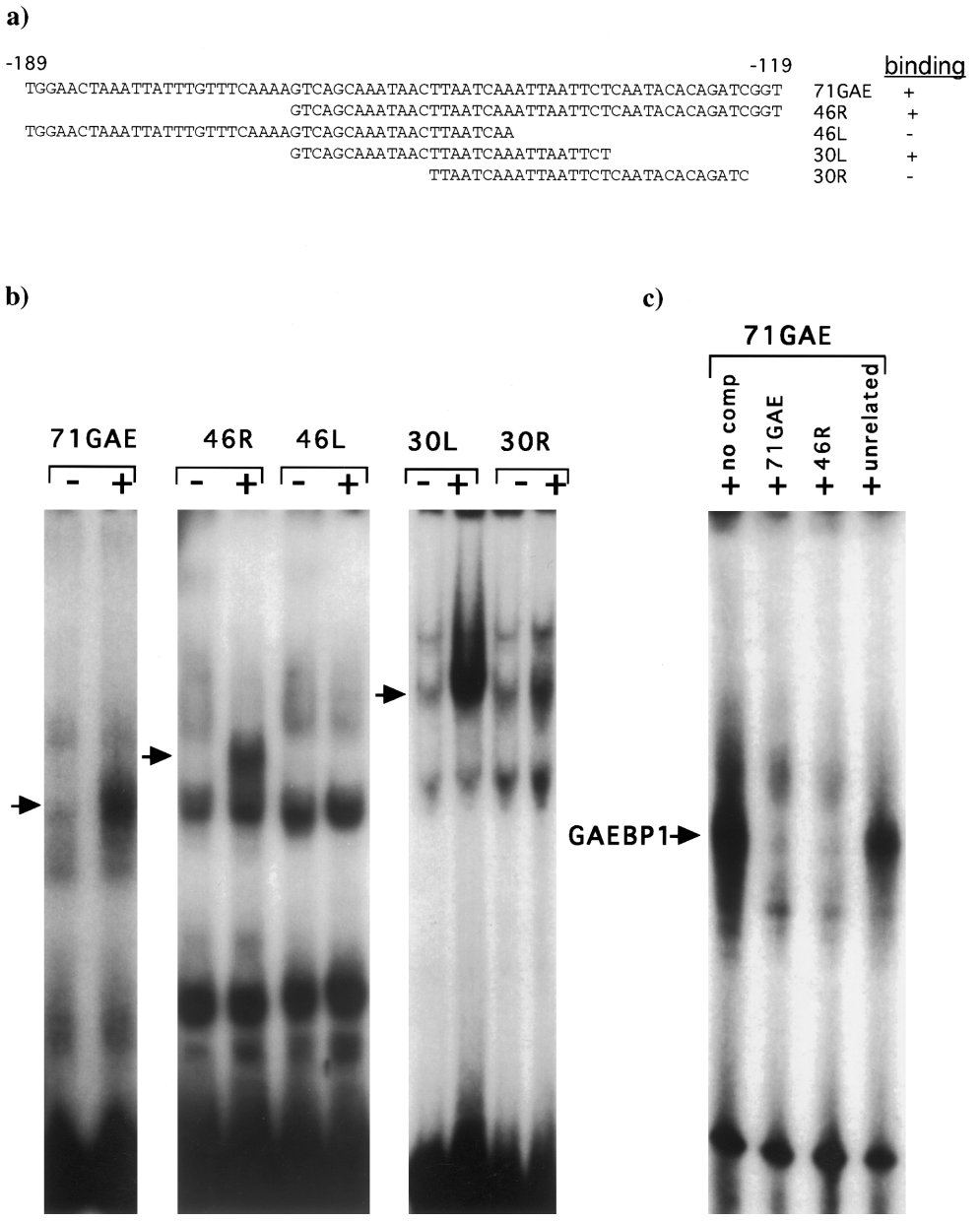

FIG. 3. The GAE binds specifically to a complex present in gastrula-stage embryos. (a) The different sequences used as probes in gel retardation assays, and their positions in the zebrafish *goosecoid* promoter, are shown. Their ability to bind an activin-inducible complex is summarized in the right-hand column. (b) Gel retardation assays were carried out on extracts from uninjected or activin mRNA-injected gastrulae. Embryo extracts were incubated with radiolabeled copies of the probes indicated in (a), and the complexes formed were separated by electrophoresis. The particular probe used is indicated above the appropriate lanes. −, extracts from uninjected stage 11 gastrulae; +, extracts from activin mRNA-injected stage 11 gastrulae. The arrows point to the position of GAEBP1. The gels with the shorter probes were not run as far, and therefore GAEBP1 is higher on the gel. (c) An extract from stage 11 activin-injected embryos was used in all lanes. The probe used was 71GAE, and extracts were preincubated with 100-fold excess of unlabeled 71GAE, 46R, or a random competitor. The 71GAE and 46R competitors efficiently compete for binding to the probe, whereas the unrelated sequences do not, indicating that GAEBP1 binds DNA specifically.

likely to be insensitive to α-amanitin. Under the conditions used in this experiment, binding of ARF was still induced by activin (Fig. 5b). Taken together, these results along with the kinetics of GAEBP1 detection indicate that GAEBP1 is not immediately downstream of activin and is distinct in its induction from ARF. It is therefore

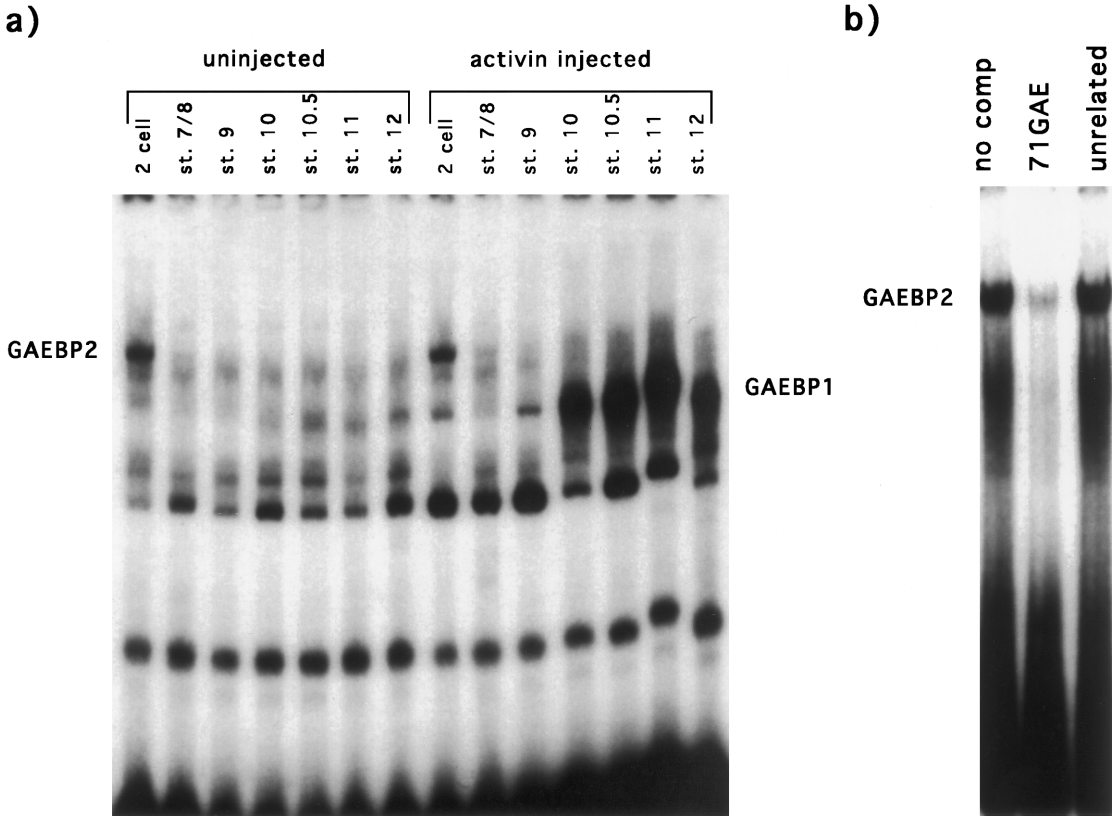

FIG. 4. Developmental time course of complexes which bind the GAE. (a) Extracts made from embryos harvested at different developmental stages were subjected to gel retardation analysis (staging is according to Nieuwkoop and Faber, 1967). The probe used was 46R. Extracts from uninjected embryos were analyzed in the left-hand seven lanes, and extracts from activin mRNA-injected embryos were analyzed in the right-hand seven lanes. Two developmentally regulated complexes were detected. A maternal complex, GAEBP2, has decreased in abundance by the start of gastrulation. The second complex, GAEBP1, is upregulated at the start of gastrulation: it is present in uninjected embryos but is induced 15-fold in activin mRNA-injected embryos. (b) The maternal complex, GAEBP2, binds DNA specifically. A 100-fold excess of cold 71GAE was added to the binding reactions and efficiently inhibited complex formation, whereas 100-fold excess of random competitor did not compete efficiently. The probe in this experiment is 71GAE.

not likely to be involved in the initiation of *goosecoid* transcription. Instead, GAEBP1 is zygotically transcribed in response to activin, and it may be responsible for the maintenance of *goosecoid* expression.

It is interesting to note that other members of the TGF-β family such as Xnr1 (data not shown) and Xnr2 (Figs. 5a and 5b), can induce both GAEBP1 and ARF, indicating that they may be capable of carrying out this induction in the embryo. GAEBP1 is also induced by Smad2 (Baker and Harland, 1996), a component of the activin signal transduction pathway (data not shown).

GAEBP1 Binding Is Required for Activin-Inducibility of the goosecoid Promoter

To learn which sites within the GAE are critical for GAEBP1 binding, we tested the ability of mutant GAEs to bind GAEBP1. The same mutations were then introduced into the context of the minimal *goosecoid* promoter (i.e., in p0.3gsclux) and their effect on the activin inducibility of the promoter was tested. The minimal 30L sequence contains two distinct repetitive elements; the sequence CAAAT is repeated, as is the sequence TTAAT. Each mutant GAE carries two point mutations disrupting both copies of such repeats (Fig. 6a). The ability of a sequence to bind GAEBP1 (Figs. 6b and 6c) correlates well with the activin inducibility of a promoter containing that sequence (Fig. 6d). Mutants TGAAT and CAAAG are no longer capable of binding GAEBP1, and when these mutations are introduced into the *goosecoid* promoter it loses its activin inducibility. Mutants TAAA and CAGA retain their ability to bind GAEBP1, and promoters carrying these mutations are still activin inducible. Interestingly, in all repeats of this experiment TAAA bound GAEBP1 more strongly, and CAGA bound GAEBP1 more weakly, than the wild-type 30L sequence. When TAAA

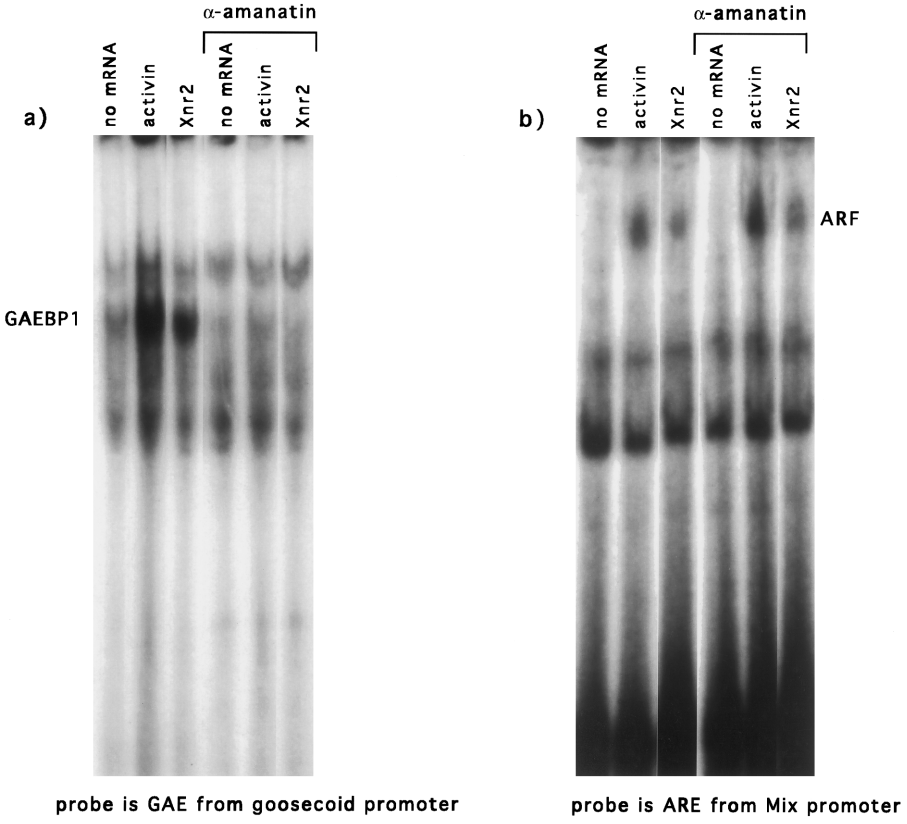

FIG. 5. Zygotic transcription is required for GAEBP1 formation. (a) GAEBP1 complex formation is induced by activin and Xnr2. Its formation is blocked in embryos injected with the RNA polymerase II inhibitor α-amanitin. Embryos were harvested at stage 11, i.e., when GAEBP1 peaks in abundance. The probe in this experiment is 30L. (b) ARF, the activin-response element (ARE) upstream of the *Mix2* gene, is still induced in the presence of α-amanitin. Embryos were harvested at stage 9, i.e., the stage at which ARF peaks in abundance.

was introduced into the promoter it became more highly activin inducible, and when CAGA was introduced into the promoter it was slightly less activin inducible, consistent with the small decrease in GAEBP1 binding. This strong correlation between GAEBP1 binding and activin inducibility indicates that binding of GAEBP1 to the GAE is required for the activin inducibility of the minimal *goosecoid* promoter.

To determine whether the GAE is significant in the context of the longer 4-kb goosecoid promoter fragment, we replaced the GAE in p4.0gsclux with a *Bam*HI recognition site and compared the activin inducibility of this mutated promoter with that of the wild-type *goosecoid* promoter. Loss of the GAE typically reduced the activin responsiveness of the 4-kb promoter by about 30- to 40-fold (Fig. 6e, constructs 1, 2, and 4 have lost the GAE and construct 3 retains the GAE). This indicates that the GAE is significant in the context of the 4-kb goosecoid promoter fragment and its function cannot be replaced by other sequences present within this DNA.

GAEBP1 Binding to the GAE May Contribute to Localizing goosecoid Expression on the Dorsal Side of the Embryo

Goosecoid is expressed in the dorsal mesoderm of the *Xenopus* embryo in response to activin-like signals. We have defined a regulatory element within the *goosecoid* promoter, the GAE, which confers activin inducibility upon a heterologous promoter and have also identified a factor, GAEBP1, whose binding to the GAE appears to be required for activin inducibility. To determine if the GAE and GAEBP1 could contribute to localizing *goosecoid* expression to the dorsal side of the embryo we injected the wild-type promoter construct (p0.3gsclux) into the dorsal or ventral marginal regions of four-cell embryos. We typically observed threefold more reporter activity on the dorsal side compared to that on the ventral side (Fig. 6f). The *goosecoid* promoter fragment that carried the CAAAG mutation which eliminates binding to GAEBP1 gave no differential activation (Fig. 6f). This indicates that the GAE and GAEBP1 can function at endogenous levels of activin-like

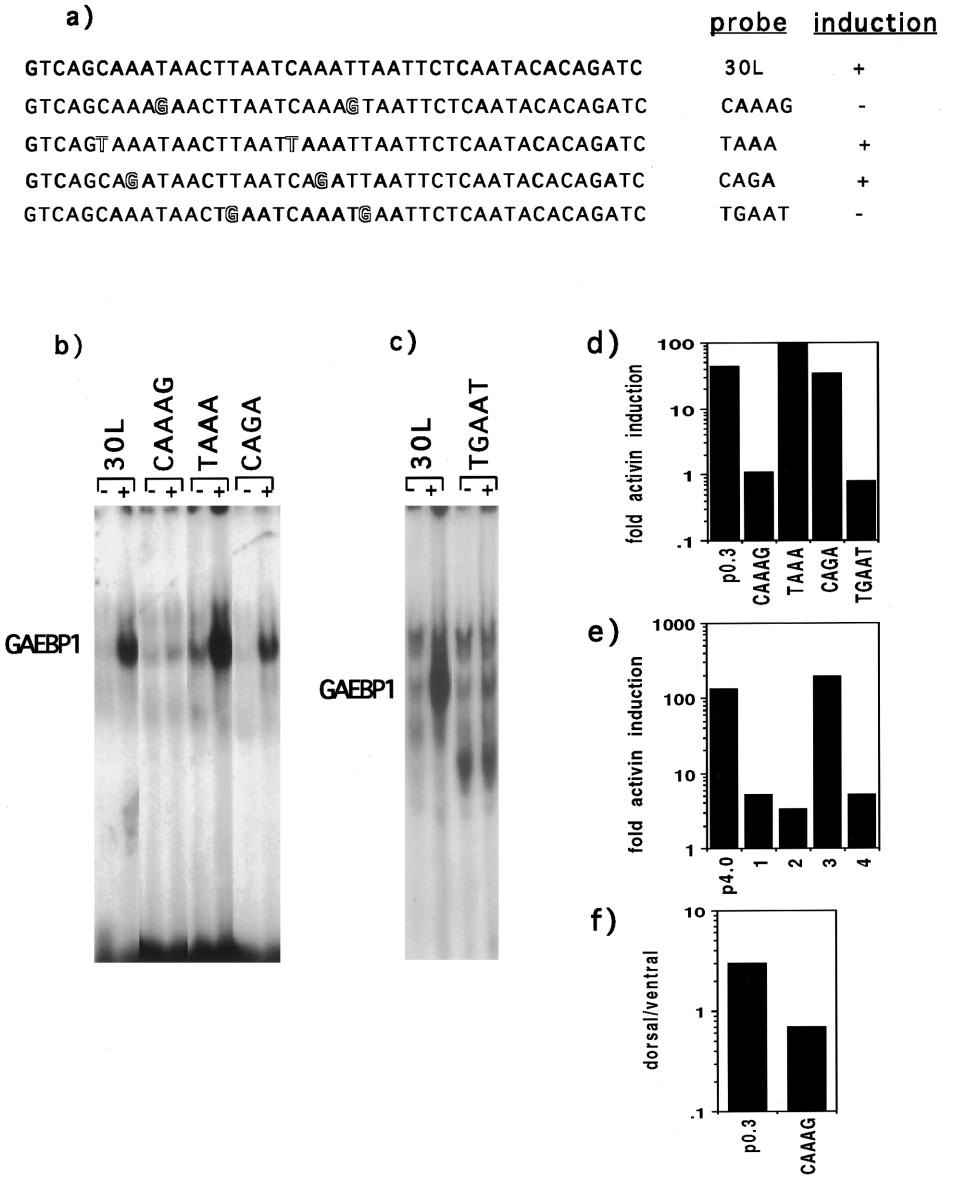

FIG. 6. Nucleotides within the GAE required for binding to GAEBP1 are also required for the activin inducibility of the promoter. (a) The point mutations introduced into the 30L sequence are indicated. Each mutant sequence carries two point mutations. (b and c) The mutant sequences were used as probes in a gel retardation assay to assess the effect of the mutations on the ability of the DNA to bind GAEBP1. The lanes in (b) are from one gel, and those in (c) are from a different gel. (d) The same point mutations were introduced into the *goosecoid* promoter and their effect on the activin inducibility of the promoter was assessed. This indicated that the nucleotides required for binding to GAEBP1 are also required for the activin inducibility of the promoter. (e) The GAE is also required in the context of the longer 4-kb promoter fragment. The GAE was deleted from the 4-kb promoter by single-strand-specific oligonucleotide-directed mutagenesis and four different products from the mutagenesis were tested. In subclones 1,2, and 4 the GAE has successfully been deleted, whereas subclone 3 retains the GAE. The results indicate that the GAE is required in the context of the longer promoter fragment. (f) The homeodomain binding site is also required for dorsal-specific transcription from the goosecoid promoter. p0.3gsclux and the promoter carrying the CAAAG mutation were injected into the dorsal or ventral marginal zones of four-cell embryos. Embryos were harvested at midgastrulation (stage 11) and the amount of luciferase activity present in dorsally injected embryos was compared to the amount of luciferase activity present in ventrally injected embryos. Wild-type *goosecoid* promoters were tested 11 times and showed differential activation on the dorsal side on 8 of these occasions. The CAAAG mutant promoter was tested three times and on none of these occasions was more active on the dorsal side. The result of a representative experiment is presented in which the wild-type promoter is three times more active on the dorsal side of the embryo. All the bar graphs are presented on a logarithmic scale.

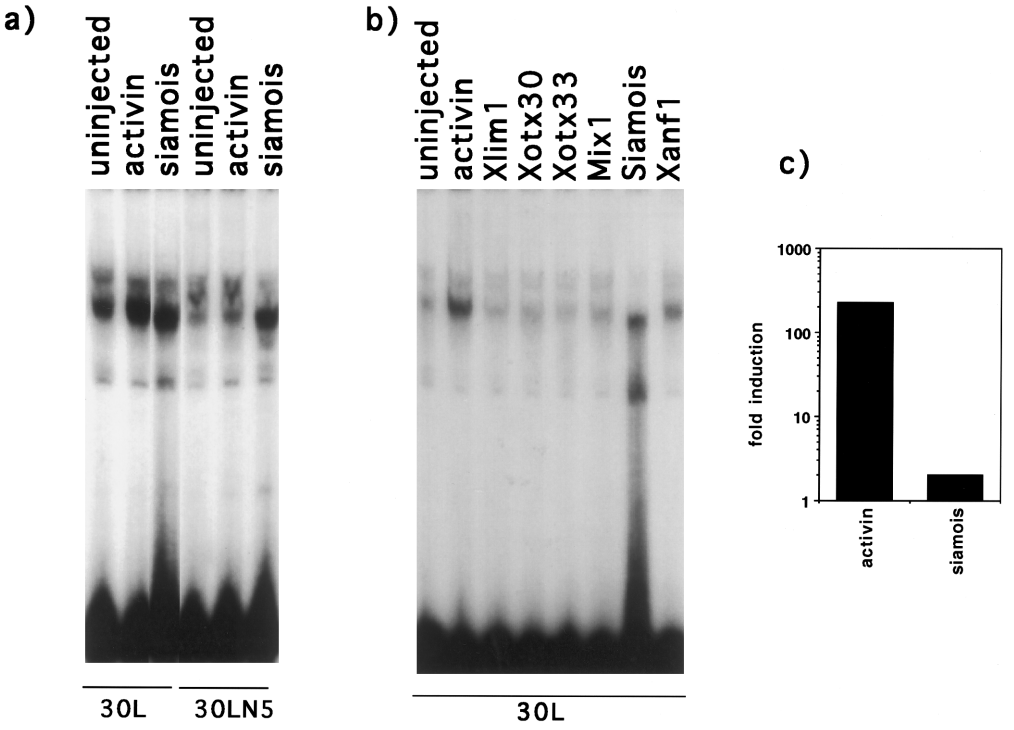

FIG. 7. Comparison of GAEBP1 with a GAE-binding complex present in *siamois*-injected embryos. (a) GAEBP1 and the siamois-induced GAE binding activity were compared for their ability to bind the short wild-type GAE, 30L, and a mutated version of this sequence (30LN5) in which an additional 2 bp were inserted between the two halves of the palindromic paired box binding site, such that the original sequence TAATCAAATTA was altered to TAATCAGGAATTA. The original 30L sequence is able to bind both GAEBP1 and the siamois-induced GAE binding activity, whereas when the two palindromic TAAT halves are separated by an additional 2 bp, GAEBP1 can no longer bind, but the siamois-induced activity can still bind. (b) 1 ng of synthetic mRNAs encoding various homeobox-containing genes was injected into *Xenopus* embryos at the single-cell stage. Protein extracts of gastrula-stage embryos were examined for the presence of GAE binding activities in a gel retardation assay. (c) Embryos were co-injected with p0.3gsclux and either 100 pg synthetic activin mRNA or 1 ng synthetic *siamois* mRNA. Extracts were made from these embryos at late gastrulation and the responsiveness of p0.3gsclux to the injected mRNA was measured as the amount of luciferase activity in mRNA-injected embryos divided by the amount in embryos that had received no mRNA. Whereas luciferase activity was highly inducible by activin, siamois consistently failed to induce luciferase expression. Results are presented on a logarithmic scale.

signals and so, may contribute to dorsal localization of goosecoid transcripts.

GAE Is a Homeodomain-Binding Site

The GAE contains a consensus binding site for the paired class of homeodomains TAATNNNATTA (Wilson *et al.*, 1993). The point mutations which specifically alter this consensus (Fig. 6a) are those which affect the functioning of the GAE. The mutant band-shift probes (Fig. 6a), which no longer bind GAEBP1, carry two point mutations. We have also tested band-shift probes which carry only one of the two point mutations. The mutations which lie within the homeodomain binding site eliminate binding of GAEBP1, whereas those which lie outside this site do not affect GAEBP1 binding (data not shown).

Binding sites for paired class homeodomain proteins contain two TAAT half-sites typically separated by 2 or 3 bp. Binding to these sites is cooperative so that when one half-site is occupied, binding to the other half-site is greatly facilitated. It has been shown that the spacing between the two TAAT half-sites is critical for this cooperativity (Wilson *et al.*, 1993). To test whether the GAE is likely to be a paired class binding site, we further separated the two halves of the GAE palindrome by inserting an extra 2 bp (thus creating TAATNNNNNATTA). This mutation eliminates binding of GAEBP1 (Fig. 7a), providing further evidence that GAEBP1 contains a paired homeodomain.

As a preliminary attempt to identify GAEBP1, we tested candidate molecules. Several homeobox-containing genes are known to be transcribed specifically within the dorsal mesoderm of the *Xenopus* gastrula, such as *Xanf1, Xlim1, Otx2, siamois,* and *goosecoid* itself (Blumberg *et al.*, 1991; Cho *et al.*, 1991; Lemaire *et al.*, 1995; Pannese *et al.*, 1995; Blitz and Cho, 1995; Taira *et al.*, 1992; Zaraisky *et al.*, 1995). Among these *goosecoid* and *Xlim1* are known to be

activin inducible, and *siamois, otx, goosecoid,* and *Xanf1* belong to the paired class. We also tested *Mix1,* a paired class homeobox gene which is expressed throughout the marginal zone and is activin inducible (Rosa, 1989), though is thought to mediate ventralizing signals (Mead *et al.,* 1996). Goosecoid itself is unlikely to be involved as it is thought to be a transcriptional repressor (Smith and Jaynes, 1996; Danilov *et al.,* 1998), but the others are candidate transcription factors for the maintenance of *goosecoid* transcription that has been induced by activin. We injected mRNAs encoding these and other homeodomain-containing proteins into *Xenopus* embryos and assayed their effects in two ways. First, we looked for the presence of GAE binding activities in extracts from gastrula-stage embryos, and second, we determined their ability to activate transcription of the *goosecoid* promoter through the GAE. Only *siamois* mRNA-injected embryos contain significant GAE-binding activity (Fig. 7b). However, although siamois can bind the GAE it does not appear to activate transcription through the GAE in this assay (Fig. 7c). Furthermore, although siamois is a member of the paired-class of homeodomain proteins, the GAE-binding activity present in *siamois* mRNA-injected embryos is capable of binding the mutant sequence in which the two TAAT half-sites are separated by 5 bp (Fig. 7a). Thus siamois alone is not likely to be responsible for this induction *in vivo.* Consistent with this is the observation that *siamois* is highly inducible by wnt family members, but is not induced by activin (Brannon *et al.,* 1997; Carnac *et al.,* 1996).

DISCUSSION

An Activin Response Element in the goosecoid Promoter Interacts with Two Developmentally Regulated Proteins

We have isolated a genomic clone of the zebrafish *goosecoid* gene and used deletion analysis experiments to locate sequence elements required for the expression of this gene during gastrulation (Fig. 2). In particular, we have defined an upstream element, the goosecoid activin element, which is sufficient and necessary for the activin responsiveness of this promoter in both *Xenopus* and zebrafish embryos. Fusion of two tandem copies of the GAE upstream of a heterologous promoter confers activin inducibility (Fig. 2e), and deletion of the element dramatically reduces inducibility (Fig. 6e).

Two complexes which interact specifically with this element in a gel retardation assay have been detected in *Xenopus* extracts, GAEBP1 and GAEBP2. GAEBP2 is maternal and declines in abundance before the onset of zygotic transcription (Fig. 4). GAEBP1 is first detected in the late blastula and peaks in abundance at late gastrula stages. It is detectable in uninjected embryos, but is induced about 15-fold in extracts from embryos that have been injected with synthetic *activin* mRNA. Increases or decreases in binding of GAEBP1 to the GAE cause corresponding changes in activin inducibility, providing strong evidence that GAEBP1 has a positive role in the regulation of *goosecoid* transcription (Fig. 3).

The minimal *goosecoid* promoter (in p0.3gsclux) is preferentially activated on the dorsal side of the *Xenopus* gastrula (Fig. 6f). Preferential activation is abolished for promoters carrying point mutations in the GAE which ablate both activin inducibility and the ability of the GAE to bind GAEBP1. This indicates that dorsal activation of the short promoter is dependent on GAEBP1 binding to the GAE. Thus, the GAE may contribute to dorsal localization of *goosecoid* transcription during gastrulation.

Induction of GAEBP1 is sensitive to α-amanitin, indicating a requirement for zygotic transcription (Fig. 5). This is interesting in the light of studies using cycloheximide which have indicated that transcriptional induction of *goosecoid* is an immediate-early response to activin treatment (Cho *et al.,* 1991; Watabe *et al.,* 1995). Our results indicate that GAEBP1 is likely to be required for the subsequent maintenance of *goosecoid* transcription in the embryo. It is possible that an initial protein synthesis-independent induction also acts through GAEBP1, and the levels of GAEBP1 are too low for us to detect. Alternatively, there is evidence of other activin-responsive elements upstream of the GAE in the zebrafish promoter, and these may be responsible for the initial induction (Joore *et al.,* 1996; S.E.S. and R.M.H. unpublished results).

Other sequence elements mediating activin inducibility have been identified. The ARE of the *Mix2* gene is required for activin inducibility (Huang *et al.,* 1995). There is no apparent similarity between this element and the GAE, and the ARE cannot compete for GAEBP1 binding in a gel retardation assay (data not shown). An activin-inducible ARE binding activity, the activin response factor, is maternally encoded and has been shown to contain a novel winged-helix transcription factor along with activated Smad2 (Chen *et al.,* 1996).

The GAE was isolated from the zebrafish *goosecoid* promoter and is activin responsive in both zebrafish and *Xenopus* embryos. The promoters are divergent in sequence but can be aligned in the region reported to contain an activin-responsive element. In zebrafish, the element TAATCAAATTA lies between −149 and −139 upstream of the promoter, while in *Xenopus,* TAATCAGATTA lies between −223 and −213 (the distal element, Watabe *et al.,* 1995). The *Xenopus* distal element was reported to confer activin induction in the presence of cycloheximide, suggesting that induction through this element is independent of protein synthesis. While the sequence of the *Xenopus* element is slightly different, it can compete efficiently for GAEBP1 binding in a gel retardation assay. It is possible that a small amount of GAEBP1 present in the embryo prior to MBT is required to initiate *goosecoid* transcription and to autoregulate its own transcription. However, while in the context of the *Xenopus* gene this element may confer an immediate-early response to activin, we suggest that, like the zebrafish GAE, it also contains the information neces-

sary to maintain *goosecoid* expression through binding of paired homeodomain transcription factors.

Experiments with heterologous promoters suggested that additional sequences might be required for the response to activin. Although two copies of the region from −189 to −119 of the *goosecoid* promoter (which contains the GAE) were sufficient to confer activin inducibility upon a heterologous promoter, in all repeats of this experiment, the heterologous promoter construct was consistently less inducible than the wild-type *goosecoid* promoter (Fig. 2e). The loss of inducibility in the heterologous promoter could be due to the different spacing between the GAE and the basal promoter elements. Alternatively, or in addition, it could represent a requirement for additional sequences for a maximal response to activin. Subsequent experiments have shown that deletion of 27 bp between −101 and −128 almost completely abolishes the activin responsiveness of the promoter (unpublished results, R.McK. and R.M.H.). This region lies outside the minimal region required for binding of GAEBPs, indicating that other sequences are likely to be involved in the activin responsiveness of the *goosecoid* promoter.

The GAE Has a Consensus Binding Site for Paired Homeodomain Proteins

We have defined a 30-bp sequence, within the GAE, sufficient for GAEBP1 binding. This sequence contains a consensus binding site for the paired homeodomain-containing proteins, TAATCAAATTA. Point mutations which specifically alter this consensus eliminate the ability of the sequence to bind GAEBP1 and simultaneously eliminate the activin inducibility of the *goosecoid* promoter (Fig. 6). This suggests that maintenance of *goosecoid* transcription in response to activin-like signals involves a DNA binding protein with a paired homeodomain.

Homeodomains have three α-helices and the third helix recognizes a core TAAT sequence. The ninth residue of this recognition helix also interacts with the two bases immediately 3′ of this TAAT core, which are variable and provide some specificity to the interaction (Treisman *et al.*, 1992). However, many different homeoboxes are capable of recognizing the same 6-bp sequence. Various mechanisms which confer further specificity to the binding have recently been described. The paired class can achieve specificity by cooperative binding to a palindromic sequence in which two TAAT half-sites are separated by either 2 bp (i.e., TAATNNATTA) or 3 bp (i.e., TAATNNNATTA) (Wilson *et al.*, 1993). It has been shown that the spacing between the two TAAT half-sites is critical for cooperative binding (Wilson *et al.*, 1993). In support of the possibility that GAEBP1 contains a paired class homeodomain we have shown that altering the separation of the two TAAT half-sites within the GAE eliminates binding of GAEBP1 (Fig. 7).

The paired class can be divided into two groups, paired and paired-like (Duboule, 1994). Paired class proteins, originally defined by their first member, paired, have a DNA binding motif in addition to the homeodomain, called the paired box. The second group, the paired-like proteins, do not have a paired box, but their homeodomain has high sequence identity to those of the paired class. It has previously been shown that paired proteins preferentially bind sequences in which the two TAAT half-sites are separated by 2 bp, whereas paired-like proteins preferentially bind sequences in which the two TAAT half-sites are separated by 3 bp (Wilson *et al.*, 1993). Mix1, Xanf1, goosecoid, siamois, and otx are paired-like proteins and therefore are good candidates to bind the GAE sequence.

We have tested some of the known homeodomain proteins that are expressed in embryos for their ability to bind the GAE and activate transcription. While siamois binds to the element, it does not activate transcription in our assay. In addition, siamois is not significantly induced by activin, but instead is highly inducible by wnt family members (Carnac *et al.*, 1996). Since the GAE does not confer wnt inducibility (data not shown), siamois does not readily fit the characteristics expected for a paired class homeodomain protein that may activate transcription from the GAE. It is somewhat surprising that otx2 was not active in our assays as it has previously been reported to induce *goosecoid* transcription (Blitz and Cho, 1995). It remains possible that GAEBP1 is a complex containing one of these known homeodomain-containing proteins. In this case we would not necessarily detect an increase in GAEBP1 binding after injection of just one of the partners of the complex.

Goosecoid itself is also a paired homeodomain protein, and the activin element we have identified has independently been identified as a goosecoid binding element (Danilov *et al.*, 1998). However, goosecoid functions as a transcriptional repressor in the context of both the goosecoid promoter (Danilov *et al.*, 1998) and the brachyury promoter (Artinger *et al.*, 1997; Latinkic *et al.*, 1997), so is not a candidate for the activin-inducible transcriptional activators. The identification of the GAE as a target of both transcriptional repressors and activators suggests the potential for complex regulation of the *goosecoid* gene. However, in the context of our experiments, in which high doses of activin induce endogenous goosecoid transcripts to high levels, the transcriptional activation by GAEBP1 must be dominant over any potential repression by goosecoid protein.

Since none of the paired homeodomain candidates fulfilled our criteria for proteins that activate transcription through the GAE, we intend to use other approaches to clone the gene(s) encoding GAEBP1. We have subjected extracts from activin mRNA-injected gastrulae to a preliminary round of affinity purification, and GAEBP1 remains intact through the purification. Since a large number of eggs can readily be injected with activin mRNA to induce the expression of GAEBP1, it should be possible to purify GAEBP1 on a preparative scale for microsequencing and molecular cloning.

The consensus binding site for paired homeodomain proteins has been determined by the SELEX method (Wil-

son *et al.*, 1993), and the *Drosophila eve* promoter is known to contain a prd binding site which is required for regulation of *eve* expression (Hoey and Levine, 1988; Fujioka *et al.*, 1996). The element that we have defined in zebrafish *goosecoid* provides an example of a vertebrate target of a paired homeodomain protein.

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